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Antithrombin activity of the hirudin N-terminal core domain residues 1–43

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Hirudin N-terminal core domain residues 1–43 (*r*-Hir$^{1-43}$) were prepared from limited proteolysis of recombinant hirudin by *V8 Staphylococcal protease* followed by purification with reversed-phase HPLC. *r*-Hir$^{1-43}$ lacks the putative reactive site of hirudin (Lys^{47}), but binds to thrombin (with K_i of 300 nM) and blocks the catalytic activity of the protease. The structural element which accounts for the thrombin inhibitory activity of *r*-Hir$^{1-43}$ is analyzed in this report.

Hirudin; Hirudin fragment; Hirudin-thrombin interaction

1. INTRODUCTION

Hirudin is the most potent thrombin-specific inhibitor known [1,2]. This inhibitor was first isolated from the leech *Hirudo medicinalis* [3] and can be now produced by recombinant DNA technology [4,5]. The exceedingly high binding affinity and specificity of the hirudin-thrombin complex has evoked intense interest in elucidating their interaction at the molecular level. Two domains of hirudin have been identified to bind specifically to independent sites of thrombin. The acidic C-terminal domain (residues 52–65) has been predicted [6] and proven [7,8] to bind to a non-catalytic site of thrombin which is required for fibrinogen recognition [9,10]. Seven lysyl residues of thrombin were recently identified to participate in this recognition site [11,12].

The N-terminal domain (*r*-Hir$^{1-52}$) blocks the active site of thrombin [13]. Within the N-terminal domain, there are three lysines (Lys^{27}, Lys^{36} and Lys^{47}), and Lys^{47} was thought to be the reactive site [2,6]. Site-directed mutagenesis subsequently confirmed a role for Lys^{47}, but at the same time also demonstrated that its contribution to the overall hirudin-thrombin binding was marginal. Replacement of Lys^{47} by either Gln [14], Glu [15] or Asn [16] caused only a ten-fold increase of the dissociation constant. Indeed the more important structural constituent of hirudin which blocks the active site region of thrombin appears to be a hydrophobic segment which is complementary to an apolar binding pocket adjacent to the catalytic center of thrombin [10,17–19]. Although the region encompassing residues 40–48 was suggested to play a role, the composition and exact location of this hydrophobic region is not known. In this communication, we demonstrate that part of this hydrophobic segment is located within the fragment containing residues 1–43 (*r*-Hir$^{1-43}$).

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2. EXPERIMENTAL

2.1. *Materials*

Human alpha-thrombin was purchased from the Center for Diagnostic Products (Boston, USA). Recombinant desulfated hirudin (CGP-39393) has been produced by Ciba-Geigy in collaboration with Platorgan KG (Bad Zwischenahn, FRG) [4] and was kindly provided by Dr W. Maerki. *N*-α-Acetylated-Hir$^{43-52}$ (CGP 48389) was kindly supplied by Dr H. Rink. *Staphylococcus aureus* strain V8 protease was from Sigma. A monoclonal antibody raised against *r*-hirudin (MAb 4049-83-12) was produced and characterized as described in [20]. The substrate tosyl-Gly-Pro-Arg-pNa (Chromozym TH) was from Boehringer Mannheim, FRG.

2.2. *Digestion of* r-*hirudin and* r-Hir$^{1-43}$ *by V8 protease*

To prepare *r*-Hir$^{1-43}$, *r*-hirudin (100 μg) was mixed with 30 μg of V8 protease in 50 μl of 50 mM ammonium bicarbonate buffer (pH 8.0, containing 2 mM EDTA). Digestion was carried out at 37°C for 1 h. The proteolytic fragments were separated by reverse-phase HPLC with the conditions described in fig.1. For extensive digestion of *r*-Hir$^{1-43}$ and its oxidized form, the samples were digested with V8 protease under similar conditions up to 24 h (with the exception of an enzyme/substrate weight ratio of 1:10). Aliquots of the time course digested sample were removed, freeze-dried and analyzed by quantitative N-terminal analysis to evaluate the extent of digestion at Glu^{8}-Ser^{9} and Glu^{17}-Gly^{18} (fig.2).

2.3. *Amino acid analysis and quantitative N-terminal analysis*

Amino acid compositions of hirudin fragments were determined by the dimethylaminoazobenzene sulfonyl chloride precolumn derivatization method [21]. N-terminal amino acids of hirudin fragments were quantitatively analyzed by the dimethylaminoazobenzene isothiocyanate (DABITC) method [22].

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2.4. *Competitive ELISA assay*

The two-step competitive ELISA, using 96-well microtiter plates coated with biotinylated *r*-hirudin complexed with avidin was described in [20].

2.5. *Amidolytic assay of thrombin*

Assays were performed as described previously [23] at 37°C in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1% poly(ethylene glycol), M_r = 6000.

3. RESULTS

3.1. *Preparation and characterization of* rHir^{1-43}

When intact hirudin was digested by V8 protease, Glu-Xaa bonds at the C-terminal region were preferentially cleaved, with Glu^{43}-Gly^{44} being the most susceptible linkage [6]. HPLC separation of the hirudin digest allowed the N-terminal core domain (*r*-Hir^{1-43}) to be isolated with purity over 99% (fig.1). The purity of *r*-Hir^{1-43} was further confirmed by quantitative N-terminal analysis (Val, >99%) and amino acid analysis (table 1).

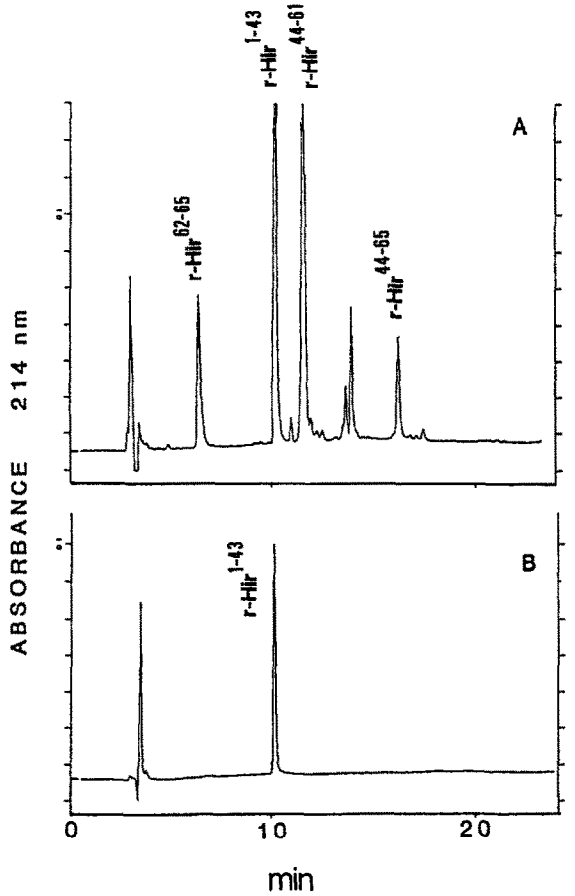

Fig.1. HPLC purification of *r*-Hir^{1-43}. (A) Separation by reverse-phase HPLC of recombinant hirudin fragments obtained after 1 h digestion by V8 protease. The peaks were identified by amino acid analysis. 100 μg of material was applied to the column. The column was Vydac C-18, 5 μm, for peptide and protein. Solvent A was 0.1% TFA in water. Solvent B was 0.1% TFA in acetonitrile/water (6:4, v/v). There was a linear gradient from 20% to 80% B in 30 min. The flow rate was 1 ml/min. (B) The peak corresponding to *r*-Hir^{1-43} was collected (from 25 repetitive injections), pooled and rechromatographed using the same conditions as in (A). 8 μg of material was applied on the column.

Table 1

Amino acid composition[a] of *r*-Hir^{1-43}

Amino acid	Found	Expected
Asp	6.23	(6)
Glu	6.80	(7)
Ser	2.95	(3)
Thr	3.22	(3)
Gly	6.76	(7)
Ala	0.10	(0)
Arg	0.17	(0)
Pro	0.06	(0)
Val[b]	2.70	(4)
Met	0.08	(0)
Ile	0.71	(1)
Leu	3.26	(3)
Phe	0.08	(0)
1/2 Cys[c]	5.90	(6)
Lys	2.10	(2)
His	0.13	(0)
Tyr	1.04	(1)

[a] 1 μg of the sample was hydrolyzed by gas-phase 6 N HCl, dabsylated and 50 ng of the derivatized sample was analyzed

[b] The lower than expected value of Val is due to incomplete digestion of Val-Val

[c] Quantitatively evaluated as cystine (2.95 residues/mol)

r-Hir^{1-43} retains the compact structure due to the intactness of the three disulfide bonds. While denatured and oxidized *r*-Hir^{1-43} was readily digested by V8 protease at Glu^{8}-Ser^{9} and Glu^{17}-Gly^{18}, its intact form was hardly digestable even after prolonged incubation (fig.2). In an ELISA assay, we have employed a monoclonal antibody to examine the cross reactivity of

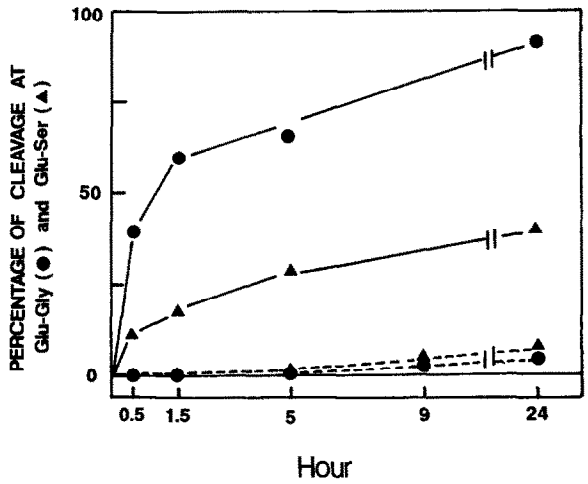

Fig.2. The digestion rate of two Glu-Xaa bonds of *r*-Hir^{1-43} by V8 protease. The intact *r*-Hir^{1-43} (dashed lines) and performic acid-oxidized *r*-Hir^{1-43} (solid lines) were digested by V8 protease under identical conditions (see text). The digestion rate of Glu^{8}-Ser^{9} and Glu^{17}-Gly^{18} was evaluated by quantitative N-terminal analysis. The recovery of the N-terminal Val was taken as 100%.

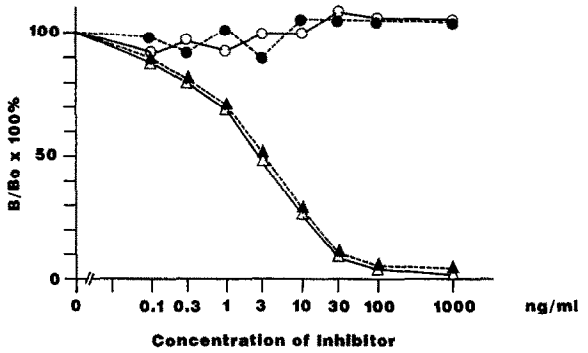

Fig.3. Competitive ELISA. Assay of MAb 4049-83-12 binding to intact recombinant hirudin (—△—), r-Hir$^{1-52}$ (—▲—), r-Hir$^{1-43}$ (—○—) and N-α-acetyl-Hir$^{43-52}$ (—●—). $B/B_o \times 100\%$ represents the percentage of MAb bound to the plate.

intact r-hirudin, r-Hir$^{1-52}$, r-Hir$^{1-43}$ and N-α-acetylated-Hir$^{43-52}$. This antibody (MAb4049-83-12) recognizes the epitope of r-hirudin at the region encompassing residues Glu^{43} to Lys^{47} [20]. The results are shown in fig.3. r-Hirudin and r-Hir$^{1-52}$ exhibit over 95% cross-reactivity. The antibody, however, does not bind to either r-Hir$^{1-43}$ or Hir$^{43-52}$. The latter result indicates that the conformation of the segment residues 43–52 within r-Hir$^{1-52}$ cannot be mimicked by synthetic peptide. This result is inconsistent with the finding that N-α-acetylated-Hir$^{43-52}$ did not display appreciable thrombin inhibitory activity even at the low micromolar concentration (data not shown).

3.2. *The thrombin inhibitory activity of* r-*Hir*$^{1-43}$

Initial velocity experiments in which both the substrate and r-Hir$^{1-43}$ were varied established that r-Hir$^{1-43}$ was a competitive inhibitor of thrombin (fig.4). These data were fitted by weighted linear regression [24] to the equation describing competitive inhibition [25] to yield an estimate of 299 ± 12 nM for the inhibition constant (K_i). This value corresponds to a binding energy of 38.7 kJ mol^{-1}.

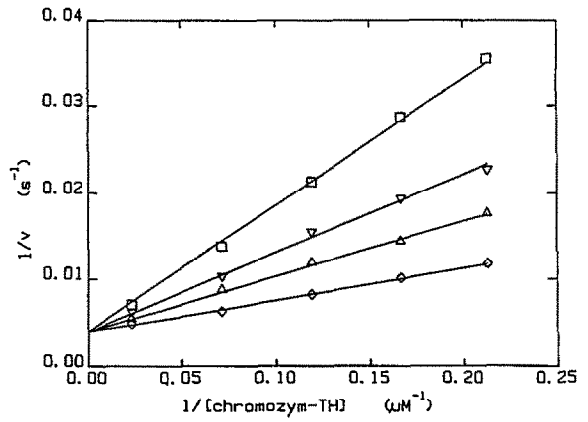

Fig.4. Effect of r-Hir$^{1-43}$ on the amidolytic activity of alpha-thrombin. Assays were performed as described in section 2 with the tosyl-Gly-Pro-Arg-pNa in the presence of 0 (◇), 225 (△), 450 (▽) and 900 (□) nM of r-Hir$^{1-43}$. The lines represent the best fit of the data to the equation for competitive inhibition; the estimates obtained from the analysis of the data were: K_{cat} = 251 ± 5 s^{-1}, K_m = 9.1 ± 0.4 μM and K_i = 299 ± 12 nM.

4. DISCUSSION

We have demonstrated that the N-terminal core domain of hirudin (r-Hir$^{1-43}$) devoid of the putative reactive site still possesses thrombin inhibitory activity. r-Hir$^{1-43}$ contains two lysyl residues (Lys^{27} and Lys^{36}), however, it is unlikely that these two lysines would contribute to its interaction with thrombin. Lys^{27} and Lys^{36} are not conserved in about 14 hirudin variants which have been sequenced so far (for review, see [26]. Replacement of them by either Gln [14], Glu or Ile [15] caused no effect on the hirudin-thrombin binding affinity. Thus, the structural element of r-Hir$^{1-43}$ which blocks the active site region of thrombin is most likely a hydrophobic cluster which is complementary to an apolar binding pocket of thrombin [10,18,19]. This apolar binding pocket is immediately adjacent to the catalytic center of thrombin [10] and makes a significant contribution towards thrombin's specificity for fibrinogen, tripeptide substrates (e.g. chromozym TH) and many nonfibrinogen polypeptide substrates [18]. The specificity of thrombin for fibrinogen is further enhanced by an independent fibrinogen recognition site of the enzyme. At least 7 lysyl residues within the B-chain of thrombin (Lys^{21}, Lys^{52}, Lys^{65}, Lys^{77}, Lys^{106}, Lys^{107} and Lys^{154}) have been identified to be involved in this fibrinogen recognition site [11,12].

As compared to r-Hir$^{1-52}$ [13], r-Hir$^{1-43}$ has a 12-fold increased dissociation constant with thrombin. This suggests that residues 43–52 contain some structural elements that interact with the active site region of thrombin. This is further supported by the observation that the monoclonal antibody (MAb 4049-83-12) which binds to this domain completely neutralizes the anticoagulant activity of hirudin [20]. Synthetic peptide Ac-Gly-Thr-Pro-Lys-Pro-CONH$_2$ (corresponding to residues 44–48 of hirudin) was also found to be a

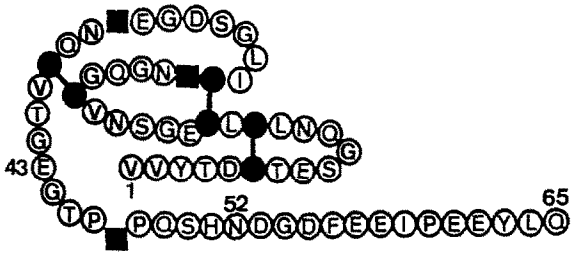

Fig.5. Structure and functional domain of recombinant hirudin. r-Hir$^{1-43}$ and r-Hir$^{43-52}$ contain structural elements which bind to the active site region of thrombin. The structural elements which bind to the active site region of thrombin are exclusively located with r-His$^{1-52}$. The structural elements which bind to the fibrinogen recognition site of thrombin are exclusively located within r-Hir$^{52-65}$. The three disulfide linkages (●—●) and three lysyl residues (■) are denoted.

moderate reversible inhibitor of thrombin with a K_i of 120 μM [29]. In addition to Lys^{47} as the putative reactive site, Pro^{46} and Pro^{48} may also form part of this hydrophobic cluster. In the structure of hirudin elucidated by NMR [27,28], Pro^{46} and Pro^{48} are close to Val^1, Val^2 and Tyr^3 [27]. Chemical modification of the N-terminal Val was quantitatively protected upon complexing with thrombin [11]. Substitution of Val^1 and Val^2 for amino acids other than hydrophobic residues caused a marked increase of the hirudin-thrombin dissociation constant [30]. Based on these data, it seems possible that Val^1, Val^2, Tyr^3, Pro^{46} and Pro^{48} are major constituents of a discontiguous hydrophobic site in hirudin that accounts for the binding strength of *r*-Hir^{1-43} and *r*-Hir^{1-52} with thrombin. The domains of hirudin which have been identified to contribute to the hirudin-thrombin complex are summarized in fig.5.

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